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Mixing of M segment DNA vaccines to Hantaan virus and Puumala virus reduces their immunogenicity in hamsters

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ABSTRACT

To determine if DNA vaccines for two hantaviruses causing hemorrhagic fever with renal syndrome, Hantaan virus and Puumala virus, are immunogenic when given in combination, we delivered them to hamsters separately or as mixtures by gene gun or by electroporation. Both vaccines elicited neutralizing antibodies when given alone but when they were delivered as a mixture, antibodies to only one of the two hantaviruses could be detected. In contrast, if the DNAs were given as separate vaccinations to a single animal, responses to both were observed. These studies suggest that the two DNA vaccines will need to be given as separate administrations.

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1. Introduction

Hantaviruses belong to the family *Bunyaviridae* and have three negative-sense genome segments, designated as L, M, and S, which encode the viral polymerase, envelope glycoproteins, Gn and Gc, and nucleocapsid protein (N), respectively. In Asia, Europe, and Scandinavia, at least four hantaviruses, Hantaan virus (HTNV), Seoul virus (SEOV), Puumala virus (PUUV), and Dobrava virus (DOBV), cause hemorrhagic fever with renal syndrome (HFRS). In America, several other hantaviruses cause hantavirus pulmonary syndrome (HPS) [1]. There are currently no U.S. licensed vaccines for hantaviral diseases, although inactivated vaccines for HFRS have been developed and tested in Asia [2]. We previously reported development and Phase 1 and Phase 2 clinical testing of a vaccinia-vectored vaccine for HFRS that expressed the M and the S genome segments of HTNV [3,4]. Although this vaccine was immunogenic in vaccinia-naïve individuals, previous vaccination with vaccinia virus greatly diminished the ability of the vaccine to elicit neutralizing antibodies to HTNV [3]. Consequently, we discontinued development of that vaccine and instead switched our efforts to generating DNA vaccines for hantaviruses.

Toward the goal of developing a DNA vaccine against all viruses that cause HFRS, we first constructed and tested plasmids expressing the M or S genome segments of SEOV or HTNV [5,6]. We found that a vaccine expressing the SEOV M segment, but not one expressing the S segment, protected hamsters from challenge with SEOV [6]. Further, we found that the HTNV M segment DNA vaccine not only provided protection from homologous viral challenge, but also elicited protective immunity to SEOV and DOBV; however, it did not offer protection against challenge with PUUV [5]. Consequently, a DNA vaccine expressing PUUV M segments was constructed and tested in rodents and nonhuman primates for use in combination with the HTNV DNA vaccine (Hooper, et al., manuscript in preparation).

Here, we report animal studies aimed at determining the feasibility of a single-combination DNA vaccine for HFRS. For this, we vaccinated hamsters by gene gun or by electroporation with the individual DNA vaccines or mixtures of the vaccines. We compared antibody responses to HTNV and to PUUV after vaccination, and we assessed the ability of the vaccines to protect the hamsters from challenge with HTNV when given alone or in combination.

2. Materials and methods

2.1. DNA vaccines

DNA vaccines expressing the M segments of HTNV [5] or PUUV (Hooper et al., manuscript in preparation) were prepared by cloning cDNA representing the viral M segments into the plasmid pWRG7077 downstream of a cytomegalovirus promoter using methods similar to those described earlier [7].

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14. ABSTRACT

To determine if DNA vaccines for two hantaviruses causing hemorrhagic fever with renal syndrome, Hantaan virus and Puumala virus, are immunogenic when given in combination, we delivered them to hamsters separately or as mixtures by gene gun or by electroporation. Both vaccines elicited neutralizing antibodies when given alone but when they were delivered as a mixture, antibodies to only one of the two hantaviruses could be detected. In contrast, if the DNAs were given as separate vaccinations to a single animal, responses to both were observed. These studies suggest that the two DNA vaccines will need to be given as separate administrations.

15. SUBJECT TERMS

Hantavirus, Hantaan, Puumala, vaccine, M-segment DNA vaccine, immunogenicity reduction, laboratory animals, hamsters

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2.2. Transfection of cells and immunofluorescent antibody assay (IFA)

COS-7 cells were seeded in four 12-well plates with coverslips in 1 ml/well of growth medium consisting of Dulbecco's Minimal Essential Medium (DMEM, GIBCO, Cat. 10569), supplemented with 4 mM L-glutamine, and 10% heat-inactivated FBS serum (FBS, Hyclone). Cells were incubated at 37 °C w/5% CO_2 until they reached ~80% confluency. Transfection of DNA plasmids was performed by adding OptiMEM (GIBCO, Cat. 31985) to a sterile tube, followed by Fugene 6 transfection reagent (Roche, Cat. 11.814.443.001) then the plasmid DNAs according to the manufacturer's directions and the tubes were incubated for 30 min at room temperature. Medium was then removed from the wells and 400 μl of fresh OptiMEM with 2% FBS was added. 100 μl of transfection mixtures was added to appropriate wells for a final DNA concentration of 2 μg/well. The plates were incubated at 37 °C in 5% CO_2 for 48 h, coverslips were removed, and processed for IFA. Medium was removed from wells and the monolayers were washed three times with 1 ml of phosphate-buffered saline (PBS). Membrane-permeabilizing fixative consisting of 50/50 methanol:acetone was added to each well, and the plates were incubated for 15–30 min at room temperature. The wells were then washed again three times with 1 ml of PBS, and the coverslips were removed from the wells with forceps and placed cell side down onto a drop of 50 μl of blocking buffer (5% goat serum in 1× PBS) on a sheet of Parafilm. The coverslips were incubated at room temperature for 15 min after which they were moved to a new drop of primary antibody prepared in block buffer. Primary antibodies used were HTNV monoclonal antibodies (MAbs) [8] 16D2 (diluted 1:200 in PBS), HCO2 (diluted 1:200 in PBS), 3D7 (diluted 1:100 in PBS). Polyclonal antibodies included a hyperimmune mouse ascitic fluid generated to HTNV or immune rabbit sera to HTNV or PUUV (each diluted 1:100 in PBS). After incubation at room temperature for 30 min, the coverslips were washed three times by sequentially dipping in beakers filled with PBS. Coverslips were then placed cell side down onto a 50-μl drop of 2° antibody (AlexaFluor goat anti-mouse or anti-rabbit Invitrogen A11001 or A11008) at a dilution of 1:1,000 in blocking buffer, and incubated for 30 min at room temperature, and then were again washed by dipping in PBS as above. The coverslips were mounted cell side down with a small drop of Prolong Gold w/DAPI (Molecular Probes, Cat. P36935) anti-fade nuclear stain. The slides were allowed to cure at room temperature overnight, and then were stored at 4 °C in the dark until examined with a fluorescence microscope.

2.3. Vaccination and challenge of animals

Animals in electroporation groups were vaccinated by the intramuscular route (i.m.) using a collared needle to deliver the DNA followed by the application of the needle electrodes of the Elgen delivery device set to a depth of 5 mm. The electrical pulses are controlled by a laptop computer with preloaded software and electroporation parameters specific to each species used. Two electroporation applications, one in each leg, were performed at each vaccination session. The groups each received three vaccination sessions at 3-week intervals. Anesthetized hamsters received 50 μg of DNA suspended in a 100-μl volume of sterile saline by needle injection in the tibialis muscle of each hind leg. Each hamster therefore received a total of 100 μg of DNA.

Animals in gene-gun groups were vaccinated intradermally (i.d.) by particle-mediated epidermal delivery (PMED). Vaccines were coated onto gold particles (~2 μm) and were delivered into epidermal cells by particle bombardment using the XR-1 delivery device (Powderject Vaccines, Inc.) as described earlier [7]. For all groups of animals, vaccination consisted of a dose of approximately 5–10 μg total (2.5 μg/shot) of each plasmid DNA. Each animal received DNA

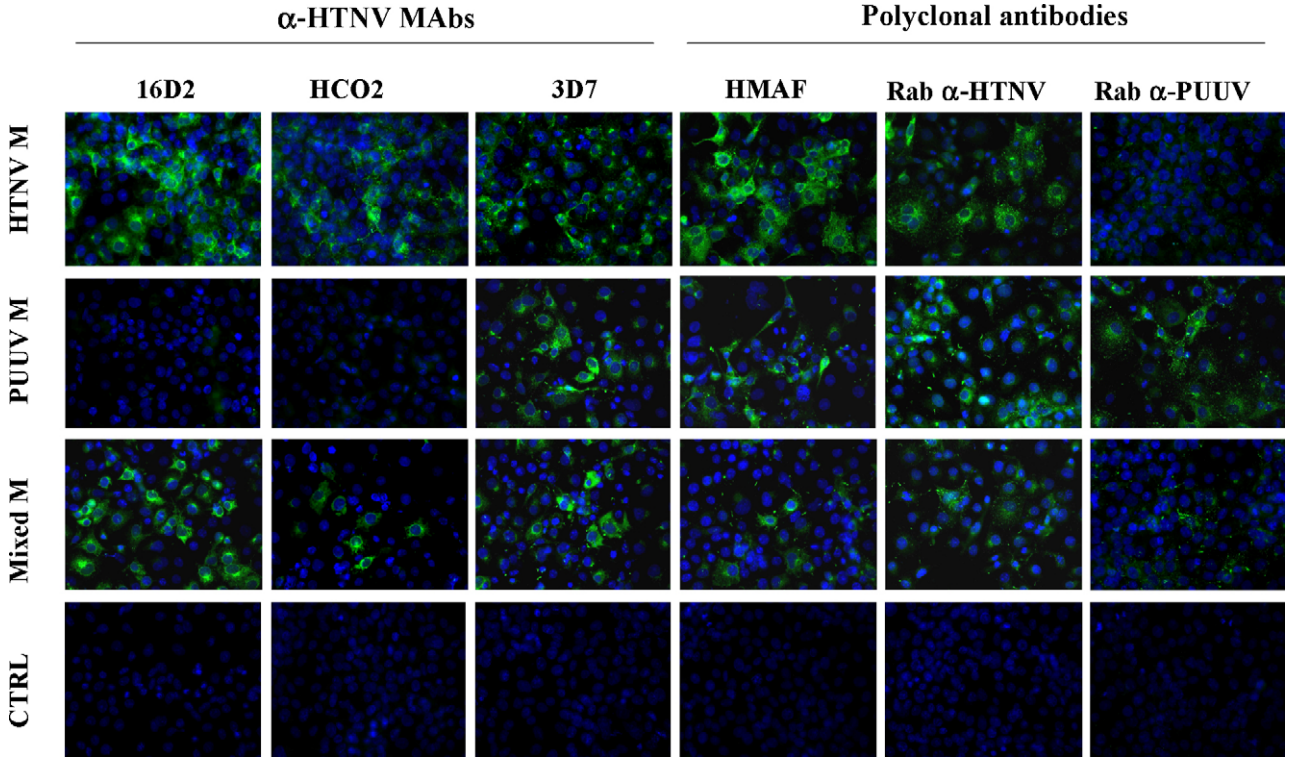

Fig. 1. HTNV DNA vaccine, PUUV DNA vaccine, or a mixture of the two DNA vaccines were transfected into cultured cells and immunofluorescent antibody tests were performed as described in Section 2. Antibodies tests included HTNV MAbs 16D2, HCO2, and 3D7 [8]. Polyclonal antibodies included a hyperimmune mouse ascitic fluid generated to HTNV (HMAF) or immune rabbit sera to HTNV or PUUV.

vaccines at two to four different sites on the abdomen at each vaccination time point. Three vaccinations were given at 3–4-week intervals.

Just before each vaccination, and 3 weeks after the final vaccination, the animals in each group were anesthetized and blood samples were obtained. Three to four weeks after the final vaccination, the hamsters in the challenge groups were inoculated with HTNV as described earlier [5]. Although hamsters do not become sick when challenged with HTNV, protection was determined by measuring antibody levels to N after challenge by ELISA.

Plaque-reduction neutralization tests (PRNT) were performed with Vero E6 cells (ATCC C1008) as described earlier [9]. ELISA was performed using *Escherichia. coli*-expressed truncated SEOV N as previously described [6,10].

3. Results

3.1. Co-expression of HTNV and PUUV M segments in cell culture

Co-expression of the HTNV and the PUUV M segment genes was compared to expression of the individual constructs by transfecting COS cells with the DNA vaccines alone or together and performing IFA. Primary antibodies used included HTNV-specific MAbs to Gn (16D2) or Gc (HCO2); a MAb to HTNV Gc that cross-reacts with PUUV Gc (3D7) [8]; hyperimmune mouse ascitic fluid (HMAF) to HTNV; and rabbit sera generated to either HTNV or PUUV. As expected, all three of the MAbs reacted with antigens in cells transfected with the HTNV M segment DNA vaccine, while only the 3D7 MAb also reacted with PUUV antigens (Fig. 1). All three MAbs also reacted to antigens in cells co-transfected with both plasmids, although fewer cells appeared to have detectable antigen than were seen in wells that had been transfected with only one of the vaccines (Fig. 1). The HMAF and the polyclonal rabbit sera to HTNV reacted most strongly to cells transfected with HTNV M segment DNA, but also reacted with cells transfected with PUUV M or both M segment DNAs. As with the MAbs, the HMAF detected fewer cells expressing antigen when transfections were performed with mixed M segments as compared to HTNV M only transfections (Fig. 1). The polyclonal rabbit sera to PUUV reacted only with PUUV antigen, which was readily detected in the PUUV M segment-transfected cells and was slightly visible in the co-transfected cells (Fig. 1). The results indicate that both plasmids expressed detectable antigen when transfected individually, and that at least one of them expressed antigen in co-transfected cells.

3.2. Immunogenicity of the individual and mixed DNA vaccines

Golden Syrian hamsters were vaccinated by PMED (gene gun) or by electroporation with the HTNV DNA vaccine, the PUUV DNA vaccine or with both vaccines. For the PMED groups, three different conditions were tested with the co-delivered vaccines: one group received separate vaccinations of HTNV or PUUV DNAs at adjacent sites; another group received both plasmids coated onto the same gold beads; and a third group received gold beads, which were coated separately with the HTNV or the PUUV DNAs, and then mixed before delivery. Control groups received the backbone plasmid with no insert. For the electroporation groups, two conditions for the co-delivered vaccines were tested: one group received both DNAs as a mixture and delivered to the same site, and another group received the DNAs at the same time, but at different vaccination sites. Comparing neutralizing antibody titers after the final vaccination revealed that both the PMED and electroporation-vaccinated hamsters developed neutralizing antibodies to HTNV or PUUV when the individual DNA vaccines were given, but that mixing the vaccines resulted in neutralizing antibody responses only to PUUV (Table 1). This effect could be overcome if both DNAs were delivered at the same time to separate sites by either method or if the DNAs were coated onto separate gold beads, and then the gold beads mixed before delivery by gene gun (Tables 1A and 1B).

3.3. Protective efficacy of the individual and mixed DNA vaccines

All hamsters were challenged with HTNV and antibody responses to HTNV nucleocapsid protein (N) (which was not part of the vaccines) were measured 4 weeks later by ELISA. As expected, the PUUV DNA vaccine was not very effective at protecting against HTNV infection, with seven of eight hamsters in the PMED group and five of eight hamsters in the electroporation group developing antibodies to HTNV N after challenge (Tables 1A and 1B). The HTNV vaccine was more effective, in that all but three hamsters in the PMED group and all but one hamster in the electroporation group showed no antibody response to HTNV N after challenge, and those that did develop antibodies had much lower ELISA titers (1:100) than were measured in hamsters that were given the control DNA (Tables 1A and 1B). As predicted by the prechallenge PRNT,

Table 1A
Pre and postchallenge antibody responses and protective efficacy of DNA vaccines delivered to hamsters by PMED

Vaccine	Prechallenge $PRNT_{50}$ GMT (range)		Postchallenge ELISA # positive (range)	% protection from HTNV challenge
	HTNV	PUUV		
HTNV DNA	640 (160–1280)	ND	3/8 (100)	63
PUUV DNA	ND	381 (<20–1280)	7/8 (800–≥12,800)	13
Both DNAs same gold	≤20 (<20–20)	349 (40–1280)	8/8 (800–≥12,800)	0
Both DNAs separate gold	1974 (320–≥5120)	44 (<20–640)	0/8	100
Both DNAs separate vacc	127 (10–≥5120)	195 (40–2560)	2/8 (200, 3200)	75
Control DNA	≤20	≤20	7/7 (12,800)	0

Table 1B
Pre and postchallenge antibody responses to and protective efficacy of DNA vaccines delivered by electroporation

Vaccine	Prechallenge $PRNT_{50}$ GMT (range)		Postchallenge ELISA # positive (range)	% protection from HTNV challenge
	HTNV	PUUV		
HTNV DNA	147 (<20–1280)	ND	1/8 (100)	88
PUUV DNA	ND	538 (80–2560)	5/8 (100–1600)	38
Both DNAs mixed	≤20 (<20–20)	247 (<20–2560)	6/8 (100–1600)	25
Both DNAs separate vacc	190 (20–1280)	640 (<20–5120)	0/8	100
Control DNA	≤20	≤20	8/8 (400–1600)	0

the mixed DNAs given as a single vaccine were poorly protective against HTNV infection with all eight hamsters in the PMED group and six of eight hamsters in the electroporation group developing antibodies to N after challenge (Tables 1A and 1B). DNAs given by PMED as a single vaccination but using gold coated with the separate vaccines before mixing protected all hamsters from infection with HTNV (Table 1A, both DNAs separate gold). Likewise, electroporation delivery of both DNAs to separate sites protected all hamsters (Table 1B). Gene-gun delivery of both DNAs to separate sites also protected the majority of hamsters from infection with HTNV as measured by the absence of detectable antibodies to HTNV N (Table 1A). One of the two hamsters in this group that did become infected and had a high postchallenge ELISA titer (1:3200) had no detectable neutralizing antibody response to HTNV before vaccination. The other hamster did have prechallenge neutralizing antibodies to HTNV, and developed a much lower response to hantaviral N after challenge (1:200).

4. Discussion

We are developing DNA vaccines to protect against HFRS caused by hantaviral infection. In earlier studies, we found that both a vaccinia-vectored M segment HTNV vaccine, and the HTNV M segment DNA vaccine included in this report protected animals against infection with three of the four hantaviruses known to cause HFRS: HTNV, SEOV, and DOBV, but did not protect from PUUV [5,11]. Consequently, our strategy was to use a combination of HTNV and PUUV M segment DNA vaccines for preventing HFRS. The easiest means for generating this type of combined vaccine was to mix the two DNAs before vaccination. To investigate that possibility, we compared the cell culture expression of individual and combined HTNV and PUUV DNA vaccines as well as their immunogenicity and protective efficacy in hamsters.

We observed expression of both the HTNV and PUUV genes in individually transfected and in co-transfected cultured cells. Because we noted that fewer cells displayed antigen when they were transfected with mixed M segment DNAs than in cultures transfected with only one or the other of the DNAs, it is possible that we were visualizing antigen only in those cells that had been transfected with one or the other of the antigens, but not both.

Our PMED results are consistent with this, in that when the HTNV and the PUUV M segment DNA vaccines were mixed then coated onto gold beads together before delivery, we observed antibody responses only to PUUV. Because expression after PMED is dependent on one or more gold beads being delivered directly into cells, preferably into the nucleus, most cells that received a gold bead would have both the HTNV and the PUUV DNAs within them. In contrast, if the individual DNAs were coated onto separate gold beads, and then the beads mixed before gene-gun delivery, some cells could get a gold bead with only HTNV DNA and others a bead with only PUUV DNA. Our results confirm that immunogenicity and protective efficacy could be elicited with this method.

Our electroporation results are also consistent with the hypothesis that when both DNAs are delivered to the same cells, an interference phenomenon is occurring. That is, if the DNAs were mixed before electroporation, poor immune responses were obtained to the HTNV antigen and there was little protective efficacy to HTNV challenge. However, if the DNAs were delivered at the same time, but to different vaccination sites, interference was no longer seen. Similar results were obtained by gene-gun vaccination of the individual vaccines to separate vaccination sites.

We did not investigate the mechanism of the interference phenomenon, but we suspect that the interference might be at the level of protein–protein interactions between the two viral envelope glycoproteins. Normally, the hantavirus M segment gene products, Gn and Gc, dimerize in the ER before transport to the Golgi for viral morphogenesis. It is possible that in cells receiving both the HTNV and the PUUV M segments, the Gn and/or Gc proteins of HTNV dimerized with the Gn and/or Gc proteins of PUUV to generate a chimeric antigen that was poorly immunogenic for HTNV. In this scenario, either these chimeras were still immunogenic for PUUV, or the PUUV Gn and/or Gc proteins preferentially dimerized with one another, thus there was sufficient non-chimeric antigen to elicit the correct immune response to PUUV.

Our findings are not without precedent. Interference was noted previously in a study with a plasmid expressing the M segment genes of both HTNV and Andes virus (ANDV), a pathogenic hantavirus from South America [12]. When delivered to hamsters separately by gene gun, the HTNV DNA vaccine was immunogenic, but the ANDV vaccine was not. When both DNA vaccines were expressed from the same plasmid, neither was immunogenic in hamsters, suggesting some sort of interference by the ANDV gene products. Interestingly, however, an immune response to both HTNV and ANDV could be obtained in nonhuman primates vaccinated with this same plasmid, indicating that animal species as well as the vaccines themselves probably play a role in the interference [12].

Several other studies with DNA vaccines have also shown that interference in vaccine components can occur. As in the present study, in one of our earlier studies, we showed that DNA vaccines representing the L1R and/or A33R genes of vaccinia virus, could individually elicit immune responses in mice, but mice given a combination of the genes coated onto the same gold beads then delivered by gene gun, developed a strong response to only one of the two genes (A33R) and were not protected from challenge. In contrast, when mice were vaccinated with the two DNAs coated on different gold beads, neutralizing antibodies (presumably anti-L1R) as well as anti-A33R antibody responses were detected, and protection was greatly improved [13].

Another recent study found strong interference when plasmids encoding different L1 genes of different papilloma virus types were used in combination to vaccinate animals. This interference could be overcome by administration of the different constructs into different sites of the animals or by sequential vaccination. The authors concluded that the cause of interference was at the level of particle assembly rather than a result of immunodominance of certain L1 proteins [14].

In contrast to these examples, several other reports (e.g., [15–17]), as well as our own studies found that it is possible to deliver combinations of certain DNA vaccines without interference with the magnitude or breadth of the immune response to the individual components. For example, we found no notable interference in immune responses elicited in animals given a combination of DNA vaccines for Ebola virus, Marburg virus, Venezuelan equine encephalitis virus, and anthrax [18]. Similarly, we did not find obvious interference in studies with combination DNA vaccines for Rift Valley fever virus, tick-borne encephalitis virus, HTNV, and Crimean Congo hemorrhagic fever virus [19]. Thus, it is clearly possible to develop certain combinations of DNA vaccines and deliver them as mixtures. However, the results reported here suggest that it may not be possible to mix the existing HTNV and PUUV DNA vaccines and give them as a single-dose vaccine. Further studies will be required to determine if the interference phenomenon can be overcome by increasing the amount of HTNV DNA in relation to PUUV DNA, although this would not be feasible with the PMED technology, as only a very limited amount of DNA can be delivered with each device. Alternatively, it might be possible to modify the genes themselves to make them more antigenic, once the mechanism of the interference is identified.

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